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RESEARCH ARTICLE

Molecular cloning, chromosomal localization and expression profiling of porcine selenoprotein *M* gene

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Abstract

Selenoprotein M may regulate a myriad of biological processes through its redox function. In pigs, neither the nucleotide sequence nor the amino acid sequence is known. Furthermore, patterns of tissue expression and regulation by dietary selenium (Se) have not been examined. We determined the full coding sequence (CDS) and the chromosomal location of the porcine gene, *SELM*, and described its expression profile *in vivo* under different dietary Se concentrations. The cDNA sequence of porcine *SELM* from the start codon to the poly(A) tail was cloned by reverse transcription PCR. The CDS contained 429 bases with a typical mammalian selenocysteine insertion sequence of form 2 (F2) located in the 3′-untranslated region. The gene was mapped to chromosome 14q21, where porcine *SELM* and its neighboring genes exhibited a similar organization to human homologues on chromosome 22q12.2. The expression pattern of *SELM* mRNA in muscle, thyroid, cerebral cortex, pituitary, testis, liver, and kidney was analyzed with real-time quantitative PCR in young male pigs fed a Se-deficient corn-soybean meal basal diet supplemented with 0.0, 0.3, or 3.0 mg Se/kg in the form of Se-rich yeast. Though the *SELM* mRNA abundance in each of the 7 tissues was not affected by the dietary Se concentrations, it was significantly higher in thyroid ($P < 0.01$) than in cerebral cortex, pituitary, testis, liver, and kidney at all of the 3 dietary Se concentrations.

Keywords Selenoprotein M; Chromosome location; Expression profile; Thyroid; Pig

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Introduction

Selenoproteins are characterized by the incorporation of selenium (Se) into the peptide as selenocysteine (Sec). With the aid of a Sec insertion sequence (SECIS) and other elements, the Sec residue is encoded by the in-frame UGA triplet that is "normally" one of the termination codons (Chambers et al., 1986). There are 25 identified selenoproteins in humans but fewer in mouse and other mammals (Kryukov et al., 2003). They are grouped into several families, including glutathione peroxidases, thioredoxin reductases, and deiodinases among other subsets. The main functions of the aforementioned families are well known, but the roles of most other selenoproteins are still unclear (Gromer et al., 2005). Selenoprotein M (SelM), encoded by *SELM*, was first described as an endoplasmic reticulum (ER) resident protein and one of the redox family members (Lescure et al., 2009). Though its antioxidant property probably contributes to the maintenance of local redox status, its specific functions are still unknown. It has long been known that the tissue expression hierarchy under different Se availability correlated with the biological importance of selenoproteins in specific tissues (Schomburg & Schweizer, 2009), so we cloned porcine *SELM*, analyzed the characteristics of its nucleotide and amino acid sequences, and investigated the tissue-specific distribution and regulation by dietary Se concentrations. Using the pig (*Sus scrofa*) as a human model (Schook et al., 2005), our results form the basis for more advanced functional studies of SelM.

Materials and Methods

Molecular cloning of porcine *SELM*

A castrated, apparently healthy 5-month-old Duroc × Landrance × Yorkshire pig was provided by Sichuan Agricultural University. It was sacrificed with the approval by the Animal Nursery Office of the university, and the loin muscle and spleen samples were immediately collected to prepare the

RNA mixture for *SELM* cloning by reverse transcriptive PCR (RT-PCR). Using the reverse transcription kit (MMLV-RT kit, Invitrogen, Part no. 28025106), the total RNA extracted with TRIzol reagent (Invitrogen, Cat. no. 15596-026) from tissue samples acted as template. The 1st-strand cDNA transcribed from the poly(A) RNA was primed by the oligo $d(T)_{18}$-adaptor primer, whose adaptor sequence (5′-CTGATCTAGAGGTACCGGATCC-3′, 3′AP) was then used as the reverse primer in the following PCR. Two specific forward primers for porcine *SELM* were designed (Primer Premier 5, PREMIER Biosoft International, Canada) based on the reference sequences obtained by *in silico* cloning using the data from National Center of Biology Information (NCBI) website. One primer was 5'-TCACCTCTAGTTTTCGGATCACCTC-3' and the other was 5'-ATGCACCTCCCGCCGCT-3'. They were paired successively with the 3′AP reverse primer in semi-nested PCR procedures using the Taq DNA polymerase kit (TaKaRa, Cat. no. DR001AM). The thermal cycling program included an initial melting at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 58~60°C for 30 sec, and 72°C for 45 sec, and a final extension at 72°C for 10 min. The amplified DNA product was analyzed by gel electrophoresis, recovered, and ligated to pMD18-T vector (TaKaRa, Cat. no. D101) by T/A cloning. After blue/white screening and sequencing (Invitrogen Company, Shanghai, China), the confirmed sequences were submitted to GenBank (ID: FJ968780).

Computational analyses and chromosome mapping

In the Gene database of NCBI, the porcine *SELM* is currently assigned to chromosome 14 (GenBank No.: NC_010456.2). Previous to the annotation, we used the *SELM* cDNA (mRNA) sequence without the poly(A) tail as the query to find the chromosomal DNA clone (GenBank No.: CT573044.1) with 100% query coverage by Blastn. This search retrieved another DNA sequence with GenBank accession no. CT954233.5. Both genomic DNA sequences bridged the gap containing *SELM* and covered more than 2600 bp on the past version of the chromosome 14 DNA sequence (GenBank No.: NC_010456.1). By *in silico* chromosome walking around porcine *SELM*, neighbor genes were identified and used to define the chromosomal location in greater detail.

The sequence of porcine *SELM* cDNA and its peptide were analyzed with NCBI Blast, SECISearch (Kryukov et al., 2003) (http://genome.unl.edu/SECISearch.html), DNAMAN 6 (Lynnon Biosoft, Canada), and MEGA4 (Tamura et al., 2007). The exons and introns of the porcine *SELM* gene were analyzed by comparing genomic DNA to the mRNA sequence with reference to the human *SELM* and the GT-AG rule (Breathnach et al., 1981).

Analysis of *SELM* mRNA expression in different tissues

To examine the gene expression profile of *SELM*, 7 tissues were sampled from young male pigs (PIC strain) fed a Se-deficient corn-soybean meal basal diet (BD) supplemented with 0.0, 0.3, or 3.0 mg Se/kg in the form of Se-rich yeast for 8-wk as described in our previous report (Zhou et al., 2009). The tissues were muscle, thyroid, cerebral cortex (of quader), pituitary, testis, liver, and kidney. The relative mRNA expression of porcine *SELM* was quantified by real-time quantitative RT-PCR (qRT-PCR) (ABI 7900HT, Applied Biosystems, USA; QuantiTect SYBR Green RT-PCR kit, QIAGEN, Cat. no. 204243, Hilden, Germany) using the ΔΔCt method and *Actb* (*β*-actin gene) as the internal reference gene. For the qRT-PCR analysis of *SELM* mRNA, the forward primer was 5'-CAGCTGAATCGCCTCAAAGAG-3' and the reverse primer was 5'-GAGATGTTTCATGACCAGGTTGTG-3'. The forward primer for *Actb* was 5'-CCCAAAGCCAACCGTGAGAA-3' and its reverse primer was 5'-CCACGTACATGGCTGGGGTG -3'.

The $\Delta\Delta$Ct values representing relative mRNA levels in all the tissues were calculated by first subtracting the average ΔCt of muscle from pigs fed BD from the ΔCt of all the tissues, and then converting the results to fold differences by raising $-\Delta\Delta$Ct to the power of two ($2^{-\Delta\Delta Ct}$). Univariate analysis of general linear model followed by Bonferroni *t* test (SPSS for Windows 13.0, USA) was used to test the effects of tissue type, Se level, and their interaction on the mRNA abundance. Data are presented as means ± standard error of the mean (s.e.m.), and the statistical significance was set at $P < 0.05$ (2-tailed).

Results

Cloning, molecular characterization, and chromosome location of porcine *SELM*

Based on the reference porcine sequences obtained by *in silico* cloning, a genomic DNA fragment of about 630 bp including the coding sequence (CDS) to the poly(A) tail of *SELM* was cloned by RT-PCR (GenBank accession no. FJ968780). The characteristics of porcine *SELM* cDNA and its SECIS element are illustrated in Figure 1. The *SELM* CDS had 429 bases with an in-frame TGA triplet; the sequence theoretically encoded 142 amino acid residues and the Sec was the 45th residue (Fig. 1A). The porcine *SELM* had 82% CDS homology to human *SELM*. According to the generally-used classification method (Grundner-Culemann et al., 1999) and SECISearch program analysis, a SECIS element of F2, with the conserved cytidine (--CC--) motif rather than the adenosine (--AA--) motif in the apical loop, was found in the 3′-untranslated region of the cDNA (mRNA), similar to that found in most mammalian species (Korotkov et al., 2002). Figure 1B compares the *SELM* SECIS elements in pig, human, mouse, and some lower animals. The SECIS element had the --AA-- motif in non-mammals, and it was F1 in some non-mammalian vertebrates like *Xenopus laevis* (frog) and *Danio rerio* (zebrafish). In contrast, some invertebrates expressed F2, including *Litopenaeus*

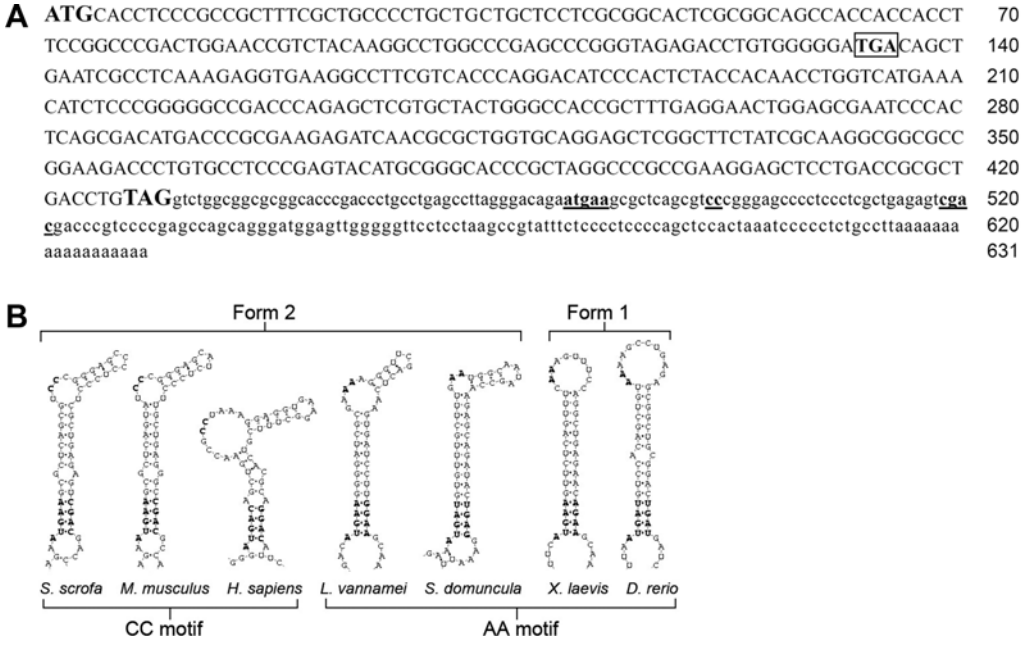

Figure 1. Porcine *SELM* cDNA and its selenocysteine insertion sequence (SECIS). A: The cDNA sequence of porcine *SELM* cloned by RT-PCR. The sequence in uppercase is the coding sequence from ATG to TAG (in bold), and the framed TGA at the 45th triplet from the initiation codon encodes selenocysteine. The underlined small letters in bold in the 3′-untranslated region indicate the highly conserved nucleotides in the SECIS. B: The SECIS structures were predicted by the SECISearch program with appropriate patterns. The highly conserved nucleotides are in bold. Accession numbers of the sequences mentioned above are FJ968780 (*S. scrofa*), NM_053267 (*M. musculus*), NM_080430 (*H. sapiens*), DQ907947 (*L. vannamei*), AJ875186 (*S. domuncula*), NM_001092248 (*X. laevis*), and NM_178286 (*D. rerio*).

vannamei (shrimp) and *Suberites domuncula* (sponge).

With the updated annotation of NCBI and the previous sequence alignment with porcine chromosome DNA sequences of CT573044.1, CT954233.5, and NC_010456.1, porcine *SELM* was localized to chromosome 14. Furthermore, by referring to the range numbers of its neighbor genes and the known exact locations of *DAO1* and *GGT1* demonstrated by previous studies (Mellink et al., 1993; Zambonelli et al., 1998), porcine *SELM* was computationally mapped to 14q21. These porcine genes were organized similarly to their homologues at human 22q12.2 (Table 1). The porcine *SELM* gene had 5 exons and 4 introns organized in the same manner as those of human *SELM* (Korotkov et al., 2002), and all the introns obeyed the GT-AG rule (Breathnach et al., 1981).

Homology and phylogeny analyses

The homology and phylogeny of porcine SelM were analyzed in several species (Fig. 2). The porcine SelM protein was aligned with 6 other mammals: *Bos taurus* (cattle), *Equus caballus* (horse), *Canis lupus* (dog), *Mus musculus* (mouse), *Rattus norvegicus* (rat), and *Homo sapiens* (human). These proteins shared 87.4% identity overall, with a highly conserved

Table 1. Chromosomal localization of porcine *SELM* and organization of its neighbor genes compared to human.

Gene symbol	Range on pig chromosome 14 (GenBank no.: NC_010456)	Location on pig chromosome 14 and evidence	Comparative location on human chromosome
DAO1	42186035-42205115[a]	14q21-23, Mellink *et al.*, 1993	7q34-q36
MORC2	48564082-48604988[a]		22q12.2
SMTN	48687102-48788249		22q12.2
SELM	48788411-48790904[a]	**14q21**, Predicted	22q12.2
RNF185	48831117-48864445		22q12.2
LIMK2	48868813-48924456		22q12.2
PIK3IP1	48925675-48936268[a]		22q12.2
GGT1	50598543-50608757[a]	14q21, Zambonelli *et al.*, 1998	22q11.23

[a] Complement in the range.
[b] Predicted by the nucleotide range and the location of its neighbor genes on pig chromosome 14.

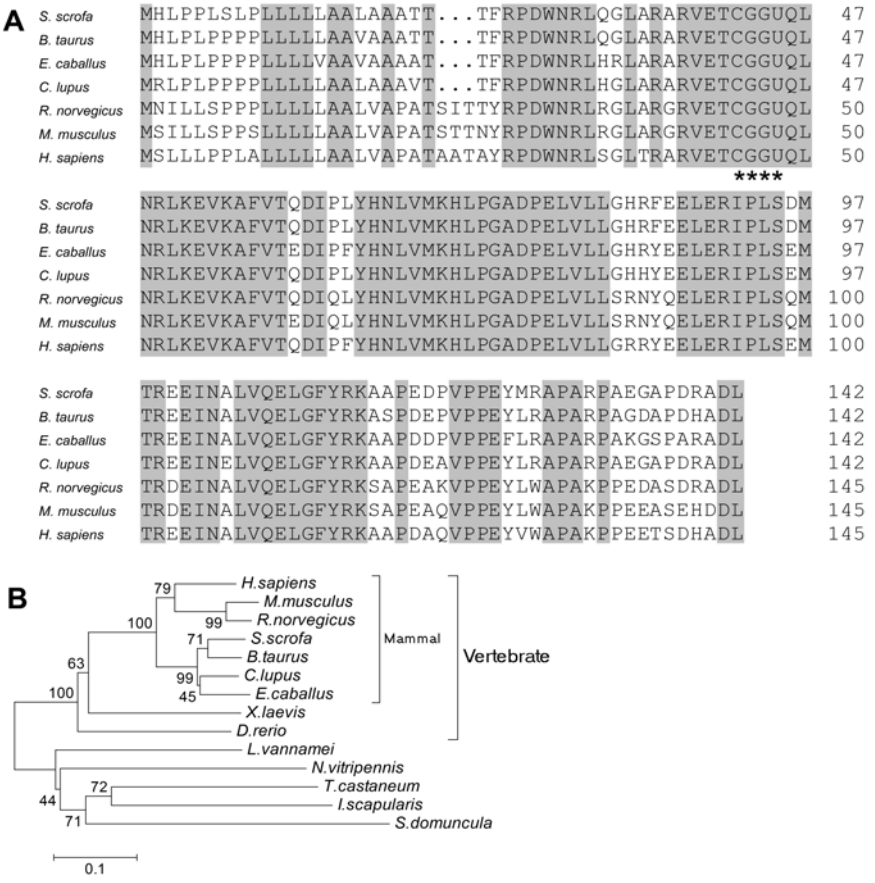

Figure 2. Alignment and phylogeny analyses of SelM based on the amino acid sequences. A: The conserved CXXU motif (CGGU in the 7 mammals) is indicated by the asterisks. The grey-colored letters indicate all conserved amino acid residues across the 7 proteins. The concatemers of proline (P), leucine (L), and alanine (A) were the dominant residues in the N-terminal signal peptide, and the H/R-X-DL was the conserved residues in the C-terminal of endoplasmic reticulum retention signal. B: The phylogenetic analyses were conducted using MEGA4 and encompassed 14 SelM proteins. The bootstrap consensus tree generated by the Neighbor-Joining method with 500 replicates and pairwise deletion options was taken to represent the evolutionary history of the 14 proteins. The statistics and frequency are presented at each of the nodes, and the length of the distance scale bar at the bottom of the panel defines 0.1 of the genetic distance.

thioredoxin-like domain containing a CXXU motif across all the 7 mammalian SeIM proteins. Proline (P), leucine (L), and alanine (A) were the dominant residues in the N-terminal signal peptide, and each usually appeared in the form of a homopolymer. The H/R-ADL was the conserved motif in the C-terminal ER retention signal, except that the mouse SelM ended with DDL (Fig. 2A). The phylogeny of SelM is illustrated by the evolutionary tree generated by MEGA4 software using *H. sapiens* and 13 other animals: *S. scrofa*., *B. taurus*, *C. lupus*, *E. caballus*, *M. musculus*, *R. norvegicus*, *X. laevis*, *D. rerio*, *L. vannamei*, *Nasonia vitripennis* (wasp), *Ixodes scapularis* (tick), *Tribolium castaneum* (beetle), and *S. domuncula* (Fig. 2B). The SelMs of the 7 mammals were genetically clustered, and of the 2 artiodactyls, pig and cattle, had the shortest distance.

Expression pattern of *SELM* in 7 porcine tissues

Univariate analysis revealed that the dietary Se concentration (in Se-deficient BD supplemented with 0.0, 0.3 or 3.0 mg Se/kg) did not affect the mRNA level of *SELM* in any of the 7 pig tissues analyzed ($P = 0.14$), but the Se concentrations in muscle were enhanced by the dietary Se supplementation ($P < 0.05$) in similar with those in liver, thyroid, and testis (OSM Table; Zhou et al., 2009). The basal mRNA expression was significantly different between tissues, however ($P < 0.01$), and no interaction effect of dietary Se concentration and tissue type on the porcine *SELM* mRNA abundance was observed ($P = 0.71$). For the *SELM* mRNA expression pattern in the 7 tissues of the pigs fed the 3 dietary Se concentrations, thyroid ranked higher than cerebral cortex, pituitary, testis, liver, and kidney ($P < 0.01$), but was not significantly different ($P > 0.05$) from muscle. In addition, muscle mRNA expression was higher than the other 5 tissues in pigs fed both BD and BD + 0.3 mg Se/kg ($P < 0.01$). When the pigs were fed BD + 3.0 mg Se/kg, *SELM* expression in muscle was statistically higher than cerebral cortex ($P < 0.01$), testis, and kidney ($P < 0.05$) (Fig. 3).

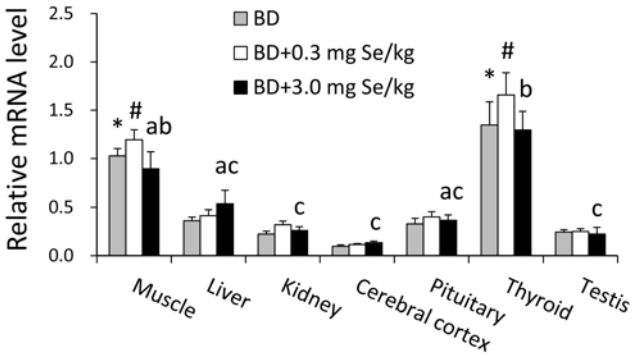

Figure 3. Abundance of porcine *SELM* mRNA in muscle, liver, kidney, cerebral cortex, pituitary, thyroid, and testis of pigs fed a basal selenium-deficient diet (BD) plus 0.0, 0.3, or 3.0 mg Se/kg for 8 wk. Data are presented as means ± s.e.m. (standard error of the mean), $n = 4$. Within the same tissue, statistical analysis found no differences under different dietary Se concentrations. At the same dietary Se concentration, means without a common symbol ($P < 0.01$) or letter ($P < 0.05$) differ between tissues.

Discussion

The *SELM* gene was first identified from the expressed sequence tags database of human and mouse by the computational method (Korotkov et al., 2002). In our study, the *in silico* method was applied to predict the CDS-containing cDNA of porcine *SELM*, and the sequence was confirmed by RT-PCR. Consistent with the *SELM* SECIS elements in human, mouse, and some other mammals, the porcine *SELM* SECIS element was of F2 and had the --CC-- sequence replacing the --AA-- in the apical loop. Substitution of adenosines for cytidines seemed to have little effect on Sec insertion into SelM (Korotkov et al., 2002). In fact, the SECIS element with the --AA-- motif appears to be universal in the *SELM* genes of lower animals, and some were even F1 (Fig. 1B). To explain the evolutionary meaning of forms and motifs in the *SELM* SECIS element, phylogenetic analysis encompassing a greater number of species is required.

Based on the available bioinformation and references, the porcine *SELM* was mapped to chromosome 14q21, in close proximity to the genes smoothelin (*SMTN*) and ring finger protein 185 (*RNF185*). The organization of *SELM* and its neighbors in pig was similar to human and mouse, consistent with the idea that entire chromosomal segments are conserved across several mammalian species (Murphy, 2003).

One study suggested that SelM probably plays a role in regulating calcium release from ER calcium stores (Reeves et al., 2010), but the molecular details remain to be elucidated. The SeIM protein was demonstrated to be a distant structural homolog (31% sequence identity) to selenoprotein 15 (Sep15) by NMR analysis, and shared many characteristics in both the proposed redox-active domain and the N-terminal signal peptide with Sep15. Moreover, both SelM and Sep15 resided in ER and were suggested to function as thiol-disulfide oxidoreductases in the quality control pathways (Korotkov et al., 2002; Ferguson et al., 2006; Labunskyy et al., 2007). The SelM protein did not have the UDP-glucose:glycoprotein glucosyltransferase-binding domain present in Sep15, however, and their functional mechanisms in the post-translational modification of ER proteins were somewhat different. When human SelM was overexpressed in a transgenic rat model, the animal had lower concentrations of H_2O_2, higher SOD and GPX activities, and a higher neutrophil-to-lymphocyte ratio. While suggesting a role for SelM in redox balance, this result provided little insight into the specific functions of endogenous SelM (Hwang et al., 2008).

The most essential selenoproteins are less affected by the Se status. In other words, not all tissues and not all selenoproteins are equally affected when Se becomes limiting (Schomburg & Schweizer, 2009). Exploring these aspects of porcine *SELM* will lead to a greater understanding of its regulation by Se status and provide clues to its biological importance in specific tissues. When SelM was first identified by Korotkov et al. (Korotkov et al., 2002), they examined its mRNA expression in 14 mouse tissues by Northern blot assay, and found that it was expressed more highly in brain than in muscle, liver, kidney, testis, and other tissues. Western blotting, however, suggested that SelM was not highly or equally expressed in all the regions of brain, and the ***cerebellar*** cortex ranked higher than the hypothalamus, isocortex, hippocampus, and olfactory bulb (Zhang et al., 2008). In our experiment, porcine tissue samples from ***cerebral*** cortex (of quader), muscle, 3 endocrine tissues, and 2 internal organs were tested by qRT-PCR. Expression was independent of changes in dietary Se and tissue Se, but basal expression of *SELM* mRNA level was significantly higher ($P < 0.01$) in thyroid and relatively lower in cortex. Moreover, the constant expression of porcine *SELM* independent of dietary Se concentrations appears to be inconsistent with the results of Sunde et al. in mouse (Sunde et al., 2009). They found that the mRNA level of murine *Selm* was moderately reduced (by 60-70% in liver and kidney) by Se deficiency, although mRNAs of more than half of the tested selenoproteins in liver and kidney were not significantly altered. These inconsistencies in tissue distribution and Se-resistance are probably due to the variations in sampling site and species. The expression pattern of porcine *SELM* mRNA was also different from that of its distant homolog, *Sep15*, where mRNA was higher in pituitary than in thyroid, testis, and liver (Zhou et al., 2009). These results implied not only the conserved function of porcine *SELM* at various dietary Se levels, but its vital tissue-specific functions in thyroid, from which hormones regulating energy metabolism (triiodothyronine and thyroxine) and calcium homeostasis (calcitonin) are generated. The pig was reported to be a better model than rodents for human thyroid hormone physiology (Wassen et

al., 2004), indicating that functional studies of *SELM* in pig thyroid may shed light on SelM function and regulation in humans.

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